Biochimica et Biophysica Acta 1828 (2013) 1700–1706

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Conformations of double-headed, triple-tailed phospholipid oxidation lipid products in model membranes

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ARTICLE INFO

Article history:
Received 6 December 2012
Received in revised form 12 March 2013
Accepted 28 March 2013
Available online 6 April 2013

Keywords:
Phospholipid oxidation
Schiff base lipid
Molecular dynamics simulation

ABSTRACT

Products of phospholipid oxidation can produce lipids with a carbonyl moiety at the end of a shortened lipid acyl tail, such as 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC). The carbonyl tail of POVPC can covalently bond to the free tertiary amine of a phosphatidylethanolamine lipid in a Schiff base reaction to form a conjugate lipid (SCH) with two head groups, and three acyl tails. We investigate the conformations and properties of this unique class of adduct lipids using molecular dynamics simulations, and show that their insertion into lipid bilayers of POPC increases the average cross-sectional area per lipid and decreases bilayer thickness. Significant increase in acyl tail fluidity is only observed at 25% SCH concentration. The SCH occupies a larger area per lipid than expected for a lipid with three acyl tails, owing to the interfacial location of the long spacer between the two head groups of the SCH. Schiff base formation of lipids can alter the concentration, homeostasis and localizations of phosphatidylserine and phosphatidylethanol lipids in membranes, and can therefore influence several membrane-associated processes including fusion and budding. The current work provides the first detailed structural model of this unique new class of lipids that may have important roles to play in modulating membrane properties and cell physiology.

1. Introduction

The polyunsaturated *sn*−2 fatty acyl chains of glycerophospholipids in lipoproteins and membranes are prone to modification by reactive oxygen species. Extensive oxidation leads to the formation of truncated phospholipids that still contain a long hydrophobic fatty acid in position *sn*−1 but have replaced the natural fatty acid in position *sn*−2 by a shorter tail with a hydrophilic terminal functional group. The ω-ends of the latter may consist of a variety of functional groups including aldehyde and carboxylic acid residues. Typical truncated phospholipids have been isolated from biological sources (*e.g.* oxidized lipoproteins, atherosclerotic plaques) and can meanwhile be prepared by chemical synthesis. The most widely investigated compounds are oxidation products of 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC) and 1-palmitoyl-2-linoleoyl-phosphatidylcholine (PLPC). PAPC generates 1-palmitoyl-2-valeroyl-phosphatidylcholine (POVPC) and 1-palmitoyl-2-glutaroyl-phosphatidylcholine, whereas PLPC is a source of 1-palmitoyl-2-oxononanoyl-phosphatidylcholine (PoxnoPC) and 1-palmitoyl-2-azelaoyl-phosphatidylcholine (PazePC). The minor structural differences between the aldehydo (POVPC and PoxnoPC) and the respective homologous (PGPC and PazePC) glycerophospholipids lead to significant changes in biophysical and biochemical properties. *In silico* studies [1] on the molecular dynamics of oxidized PazePC and PoxnoPC have revealed that the fragmented acyl chains are expelled from the hydrophobic regions of the bilayer. Whereas the carboxyl residue of the former lipid protrudes into the aqueous phase, the less polar aldehyde function resides in the lipid–water interface of the bilayer (monolayer) [2–4]. As a consequence, the respective structural motifs can interact with both the molecules in the same supramolecular assembly as well as with (cell, lipoprotein) surfaces from outside [5]. The lipid conformations seem to be very important for the bioactivities of the respective compounds, since the biological effects of truncated phospholipids depend to a much larger extent on the fragmented acyl chains than on their polar head groups [6]. The carboxylate phospholipids can only physically interact with the biomolecules in their immediate vicinity. In contrast, phospholipid aldehydes can undergo chemical reactions with free amino groups of proteins and aminophospholipids (phosphatidylethanolamine and serine) (Fig. 1) by Schiff base formation [7]. Substantial evidence is already available from experiments *in vitro* and *in vivo* showing that protein modification by aldehydolipids can lead to a plethora of functional and biophysical consequences including association with membranes, aggregation, gain and loss of function [7,8]. 4-Hydroxynonenal is currently the most profoundly studied lipid oxidation product in this respect [9]. Less information is available about phospholipid aldehydes [7]. Kinnunen and colleagues provided evidence that modification of

Abbreviations: MD, molecular dynamics; SCH, Schiff base lipid; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; QM, quantum mechanics; SI, supplementary information

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http://dx.doi.org/10.1016/j.bbamem.2013.03.030

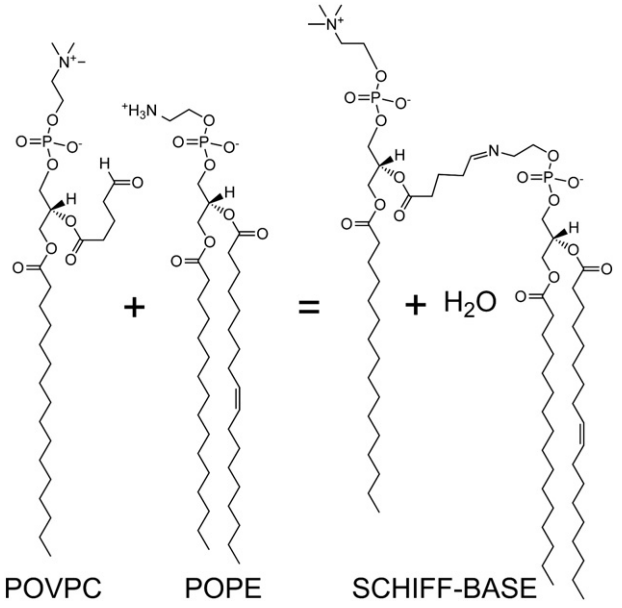

Fig. 1. The Schiff base condensation reaction between a POPE lipid and an oxidized POVPC lipid.

enzymes and antimicrobial peptides improves association with artificial membranes [10]. In a recent study, primary protein targets of a fluorescent POVPC have been identified in cultured RAW macrophages [11]. In order to verify their functional roles in the cells, these polypeptides are currently subject to biophysical (spatial lipid–protein proximity) and biochemical (gain and loss of function) studies in live cells.

Information about the (supra)molecular properties of Schiff bases formed between phospholipid aldehydes and aminophospholipids is still scarce for two reasons. First of all, such compounds have not been isolated yet from biological sources. They are labile and require stabilization before isolation, especially if the natural lipid content is low. Secondly, defined biophysical studies require chemically defined lipids. Efficient methods are still not available for synthesis, and thus, we are developing procedures for the preparation of such compounds. Therefore, *in silico* studies are for the moment the only way to get an idea of how such follow-up products of phospholipid oxidation might behave. If an aminophospholipid containing two hydrophobic acyl chains reacts with an oxidized phospholipid, an amphipathic compound with a large hydrophobic moiety and a less polar head group will be formed. This may lead to significant changes in membrane organization and changes in bimolecular (lipid–lipid and lipid–protein) interactions. Thus, it was the aim of this study to understand the behavior of such adducts in a phosphatidylcholine bilayer as well as the consequences of adduct formation on the supramolecular properties of the bilayer itself. Here we report on a molecular dynamics (MD) study on a model Schiff base generated from POPE and POVPC. We found that substantial amounts of this unusual lipid can be incorporated in a membrane. However, lipid packing and membrane thickness are significantly altered. These data are relevant not only to effects of oxidized phospholipids on membrane organization but also to functions of membrane proteins that critically depend on these parameters.

2. Materials and methods

Simulations of the Schiff-base lipid (SCH) in POPC bilayers were carried out with SCH:POPC ratios of 1:16, 1:8, 1:4 and 1:1. Below, the force-field derivation for the Schiff-base is described, followed by the simulation protocols.

2.1. Force field parameters

The modified Berger force field [12], with parameters adapted from http://moose.bio.ucalgary.ca/ was used for POPC and POPE. Water was represented by a single point charge (SPC) water model [13]. The parameters around the C═N bond were obtained using standard protocols on the parent compound shown in Fig. 2. In short, the structure was geometry optimized using Gaussian 03 [14] at the B3LYP/6-311++G** level of theory. The electrostatic potentials were sampled for each molecule at a number of points at the HF/6-31G* level of theory and the atomic partial charges were calculated using an electrostatic potential (ESP) fit. The optimized structures had their non-polar hydrogen atoms combined to the attached carbon atoms to create the united atom structures. The nitrogen atom was not protonated. The final partial charges are shown in Fig. 2. Atoms were assigned atom types from the Berger force field whenever possible.

2.2. System construction

The pure POPC bilayer contained 64 lipids in each leaflet. The bilayer had been pre-equilibrated and simulated for approximately 100 ns. The initial rectangular box sizes were 64.07 Å × 64.07 Å × 88.82 Å. Equal numbers of POPC lipids were replaced with SCHs from each leaflet. The initial conformation of the SCH molecules was such that the palmitoyl tail of the POVPC moiety pointed into the aqueous phase. The bilayer simulations will be referred to as BIL8, BIL16 and BIL32, with the trailing number corresponding to the number of SCHs in the bilayer. At higher concentrations of SCH (BIL32), the POVPC palmitoyl tails tended to aggregate in the aqueous phase, trapping the system in an intermediate state where SCHs aggregated in the lipid bilayer, and the POVPC palmitoyl tails could not insert into the lipid bilayer. For BIL32, therefore, simulations were implemented where the terminal methyl groups of the POVPC palmitoyl tails were pulled into the bilayer with a harmonic force for 50 ns. The pulling force constant was 500 kJ mol^{-1} nm^{-2}. This pulled the POVPC palmitoyl into the lipid bilayer. The forces were removed after 50 ns, and the system was simulated for at least another 1 μs. This simulation is referred to as PULL32.

The SCH carries one negative charge, and K^+ ions instead of Na^+ ions were used as counterions to avoid force-field related artifacts or bilayer condensation [15].

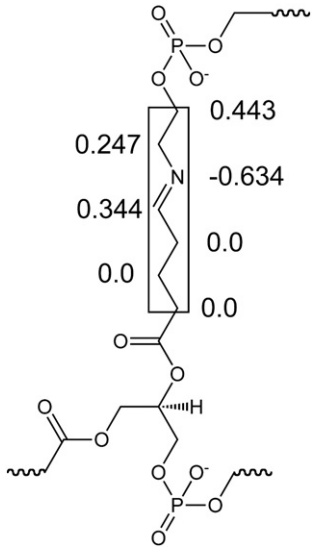

Fig. 2. The parent molecule used to develop force field parameters for the C═N bond, shown in the box. The final partial charges on united atoms are shown. The partial charges were adjusted slightly to sum up to reasonable charge groups. The nitrogen atom was not protonated.

2.3. Simulation procedure

All simulations were performed with the GROMACS package version 4.0.7 [16–19]. After energy minimization with the steepest descent method, the systems were heated during 10 ps of MD by assigning random velocities to the particles according to a Maxwell distribution at 310 K. For the production run, the leap-frog integrator [20] was used with a time step of 2 fs. All bond lengths were constrained using the LINCS algorithm [21,22], and water molecules were constrained with SETTLE [23]. Periodic boundary conditions were applied in all directions. A neighbor list with a 10 Å cut-off was used for non-bonded interactions and was updated every 20 fs. The van der Waals interactions were truncated with a cut-off of 10.0 Å and the electrostatic interactions were treated with the Particle Mesh Ewald (PME) method using default parameters [24,25]. The center of mass translation was removed at every step of the simulation. Simulations were run for at least 750 ns, and in some cases were extended to 1.5 μs (Table 1).

The *NPT* statistical ensemble was used. Temperature coupling was performed using the Berendsen thermostat [26] separately for the lipids and the rest of the system with a reference temperature of 310 K, and a time constant of 0.1 ps for both subgroups. A semi-isotropic pressure coupling was applied using the Berendsen barostat [26] with a coupling constant of 0.1 ps and a reference pressure of 1.0 bar in all directions. Since the compressibility, κ, for the systems was not known, the value for pure water, $4.5 \cdot 10^{-5}$ bar^{-1}, was used. Trajectories were sampled every 20 ps. For calculation of ensemble-averaged properties, the first 700 ns of each simulation were discarded. The analysis was carried out using GROMACS or custom-made programs. Visualization and snapshots were rendered using VMD [27].

3. Results

We will start this section by describing the conformations of the SCH in the POPC bilayer, followed by the effect of SCH on the properties of the bilayer.

The POVPC palmitoyl tails are inserted into the lipid bilayer in 260 ns and 790 ns in the BIL8 and BIL16 simulations respectively. Simulation snapshots are shown in Fig. 3. In each case, the SCHs aggregated prior to insertion, because the hydrophobic POVPC palmitoyl tails aggregated in the aqueous phase to minimize entropy loss. After the POVPC tails were inserted into the bilayer, though, the SCHs randomly diffused apart. The long time scale of insertion of the palmitoyl tails is thus a result of the collective behavior of the SCHs.

No pores were detectable through the bilayer in any BIL simulation, or in PULL32. In BIL32, the POVPC palmitoyl tails failed to insert into the bilayer spontaneously. At higher SCH concentrations, larger POVPC palmitoyl aggregates are formed in the aqueous phase, which take longer to insert into the bilayer. It may be noticed that the initial conformation of the POVPC palmitoyl tails is somewhat non-physiological, because POVPC would be typically inserted into the bilayer to begin with [1]. However, the initial states adopted in the simulations give confidence to the hypothesis that SCH can insert into lipid bilayers. In the PULL simulations, where the POVPC palmitoyl was artificially pulled into the hydrophobic core of the membrane, the SCHs remained inside the bilayer at high concentrations for up to 1.5 μs, suggesting that the SCH-aggregated state at high SCH concentrations is likely to be a local energy barrier to insertion of hydrophobic POVPC palmitoyl tails into the bilayer. In the remaining part of the manuscript, we will compare the properties of the POPC, BIL8, BIL16 and PULL32 simulations.

Table 1
Overview of the simulations performed. The bilayer had a total of 128 lipids.

Bilayer	Number of SCHs (% SCH)	Simulation time (μs)
POPC	0 (0)	0.43
BIL8	8 (6.25)	0.94
BIL16	16 (12.5)	0.95
BIL32	32 (25)	0.75
PULL32	32 (25)	1.116

In Fig. 4, the density profiles of various chemical moieties on the Schiff base and POPC are shown for the BIL16 simulation. The detailed density profiles for the phosphate, glycerol and choline groups in the BIL8, BIL16 and PULL32 simulations are shown in the Supporting information in Figs. S2, S3 and S4 respectively. From Fig. 4, it is apparent that the POPE phosphate is located closest to the bilayer center, while the POPC phosphate sits between the POPE and the POVPC phosphate groups. The same is true for the BIL8 and PULL32 simulations as well (Figs. S2 and S4). It is also apparent from these figures that the glycerol backbone of POPE is also closer to the bilayer center than the glycerol backbone of POVPC in each of the three simulations with the Schiff base lipid.

The density profiles of the POPC phosphate, choline and glycerol groups, for each of the simulations are compared in the Supporting information: Figs. S5, S6 and S7, respectively. The peak of each group is located closer to the bilayer center upon addition of the SCHs, in a concentration-dependent manner, which is a result of the overall decrease in the bilayer thickness (see below). The density profile of water for all simulations is shown in the Supporting information Fig. S8. It is apparent that with the increasing concentration of the SCHs inside the bilayer, water molecules penetrate the membrane slightly deeper. Again, this is purely a result of the reduced thickness of the lipid bilayer. As described below, the hydration level of the POPC headgroups actually decreases a bit with increasing SCH content in the bilayer.

The hydration level of the POPC headgroups was estimated by calculating Radial Distribution Functions (RDFs) between the POPC choline group and water molecules. The RDFs are shown in Fig. 5, and reveal that the Schiff lipids inclusion only slightly reduces the hydration of the POPC hydrophilic heads. The effect of the SCH molecule on POPC lipid hydration is similar to that reported for 10 mol% of POVPC [3]. SCH headgroups stick out of the bilayer (see density profiles in SI), shielding the nearest POPC molecules from water. The hydration level of the choline moieties may be reduced by the hydrophobic linker joining the two lipids in SCH, that is oriented almost parallel to the bilayer normal, analogous to the oxidized *sn*−2 chains of POVPC [3]. On the other hand, the disorder caused by SCHs in the membrane should increase water penetration and hydration of the headgroups (see density profiles for POPC in Fig. S7). The two competing factors ultimately lead only to a slightly reduced headgroup hydration.

The linker between the POPE and POVPC lipids is hydrophobic in nature except for the C═N bond, and acts as a spacer between the two lipid head groups. The spacing between the two lipid head groups forces SCH to occupy a larger area per lipid than expected for a two-headed, three-tailed lipid without a spacer. Fig. 6 shows the average area per lipid and thickness in the simulations. The projected area per lipid of the bilayer was obtained by dividing the area of the simulation box in the membrane plane by the number of lipids in one leaflet. The bilayer thickness was calculated from the distance between the two maxima in the electron density profiles of the lipids. 25% SCH increases the area per lipid to 81 $Å^2$. The area and the volume of the system have been plotted as a function of the mole fraction of the guest molecule: SCH (x_{SCH}) in Fig. 7. From basic physical chemistry, the partial molecular areas and volumes of POPC (A_{POPC} and V_{POPC}) and SCH (A_{SCH} and V_{SCH}) will be independent of x_{SCH} in ideal mixtures and a plot of the total area *versus* x_{SCH} will be a straight line [28], from the equation of which one can obtain the quantities A_{POPC}, V_{POPC}, A_{SCH} and V_{SCH}. The POPC SCH mixture is thus ideal (Fig. 7), and the partial molecular volumes of POPC and SCH are 1274 and 1900 $Å^3$ respectively. The volume of SCH is about 50% higher than that of POPC, because SCH has one extra lipid tail. The partial molecular areas for POPC and SCH are 68.2 $Å^2$ and 124 $Å^2$. The area occupied by a single SCH molecule is comparable to tetramyristoyl cardiolipin, which has four acyl tails, but has a smaller head group, and an area per lipid of 126 $Å^2$ (Richard Pastor,

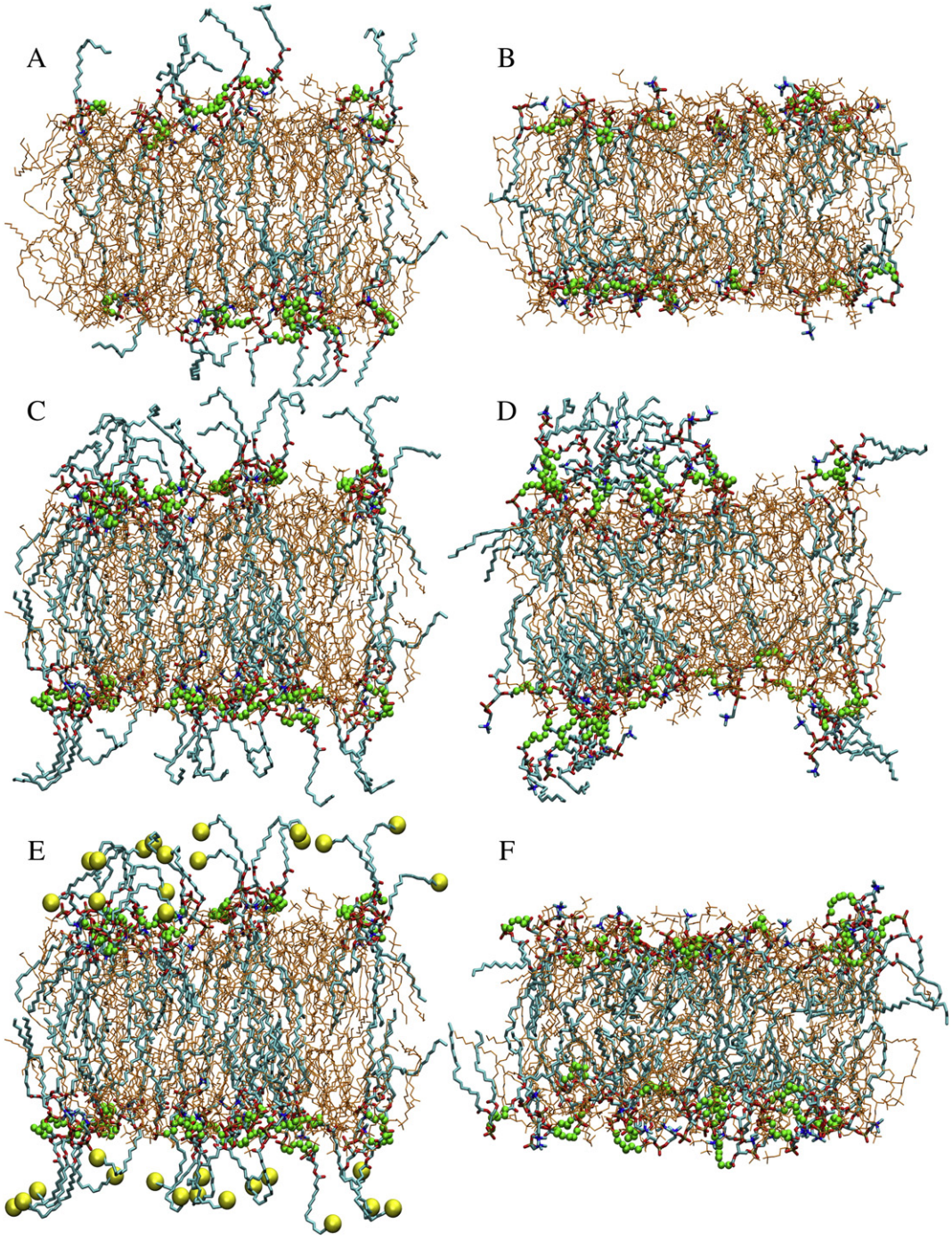

Fig. 3. The initial (A, C, E) and final (B, D, F) simulation snapshots from the BIL16, BIL32 and PULL32 simulations. POPC is shown in orange. SCH acyl tails are shown as thick cyan sticks. The spacer between the POVPC and POPE moieties is shown as green spheres, phosphate groups of SCH are shown in red and gold. Water has not been shown for clarity. In (E), the yellow spheres are the terminal methyl groups of the POVPC palmitoyl that were pulled into the bilayer for 50 ns, after which the pulling forces were removed.

personal communication). The increase in area of SCH with the additional acyl tail is much higher than 50%. The 20% larger value than expected for the 3-tail lipid suggests a disordering effect on all lipids, which is in excellent agreement with the lower orientational order parameters of bilayers doped with SCH (see below). The increase in area per lipid results from the larger head group, and the presence of only three lipid acyl tails, as opposed to four in the case of cardiolipin, should result in increased free volume in the bilayer core, resulting in a lower bilayer thickness and increased fluidity in the lipid acyl tails. Indeed, the thickness of the bilayer is decreased by 5% in the PULL32 simulation. The impact of the SCHs on the lipid bilayer properties is therefore very different from other large phospholipids.

The increased area per lipid upon addition of SCH is accompanied by an increase in the angle made by the POPC P-N vector with the bilayer normal (Supporting information, Fig. S1). For the POPE moiety, the P-N vector points in the opposite direction because the POPE nitrogen is involved in the Schiff base formation. The distribution of the P-N vector of POVPC is wider than that of POPC (Fig. S1).

The orientational order parameters of the hydrocarbon tails of the lipids were used to quantify the fluidity of the membrane. The *z*-component of the order parameter for a given vector is defined by

$$S_z = \frac{3}{2}\left\langle \cos^2\theta_z \right\rangle - \frac{1}{2}$$

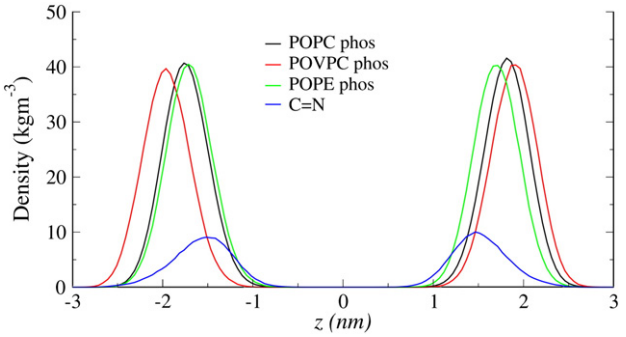

Fig. 4. Density profiles for the three different phosphate groups and the C═N bond of SCH in the BIL16 simulation. $z = 0$ corresponds to the bilayer center. Phos refers to the phosphate group of each lipid. The POPE head group is embedded deeper into the bilayer, than the POVPC head group. The POPC head-group lies in between the two. The black line is for the phosphate group of POPC. The red and green curves are the profiles for the phosphate groups on the POVPC and POPE head-groups respectively.

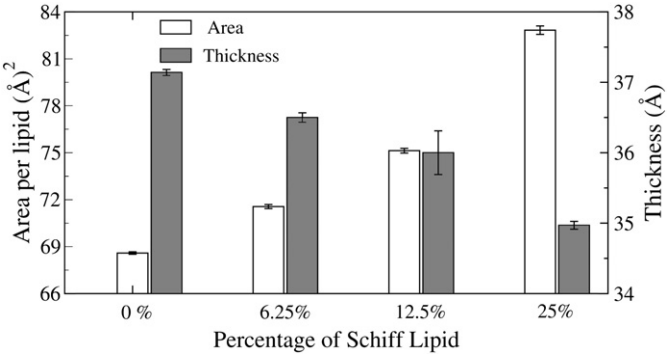

Fig. 6. The projected area per lipid and bilayer thickness. The error bars are block averages. The projected area per lipid of the bilayer was obtained by dividing the area of the simulation box in the membrane plane by the number of lipids in one leaflet. The bilayer thickness was calculated from the distance between the two maxima in the electron density profiles of the lipids.

where θ_z is the angle between the vector and the z-axis of the simulation box. The brackets imply averaging over time and molecules. An order parameter of 1 indicates a bond perfectly aligned with the bilayer normal, an order parameter of zero indicates completely random bond directions and an order parameter of $-1/2$ indicates a bond perpendicular to the bilayer normal. The order parameter (S) for the POPC palmitoyl and oleoyl tails is shown in Fig. 8, and it decreases significantly upon inclusion of 25% SCH in the bilayer.

The orientation of the acyl tails of SCH was quantified by measuring their tilt angle distributions in the bilayer. The tilt angle distributions for the three acyl tails of SCH are similar to those of POPC acyl tails, except that at larger concentrations of the SCHs, the tilt angle distribution for all tails is slightly wider (data not shown). The linker joining the two lipids in SCH lies at an angle between 81 and 86° to the bilayer normal for the three different SCH simulations.

4. Discussion

Lipid oxidation is increasingly being recognized as a key molecular process in the genesis of a large variety of disease and cellular states [8]. Lipid oxidation produces a large variety of oxidized phospholipid (OXPL) species, and many of these, which may have important roles to play in signaling, transport, and modulation of membrane properties, remain yet to be characterized [7,29]. In this work, we have characterized the molecular behavior of an exotic class of OXPLs: lipids with three tails and two heads, resulting from the Schiff base condensation between the amine of POPE and the carbonyl group of another OXPL. The exact role of SCH in disease, physiology and membrane biophysical properties is still unknown, and the present simulations construct a molecular model which can be used to interpret biophysical and biochemical data pertaining to SCHs.

In our simulations, the POVPC palmitoyl tails were initially placed in the aqueous phase, despite temporarily aggregating, spontaneously inserted into the bilayer at low (6.25% and 12.5%) concentrations on time scales of 100 s of nanoseconds, indicating that the SCH would be accommodated in membranes at low concentrations. At 25% SCH, the palmitoyl tails had to be pulled into the bilayer, but remained put in the subsequent 1.5 μs of simulations, suggesting that 25% SCH could also be accommodated. Of course, these hypotheses need to be confirmed in experiments, which can be implemented once the SCH is either chemically synthesized or isolated in large amounts from cells.

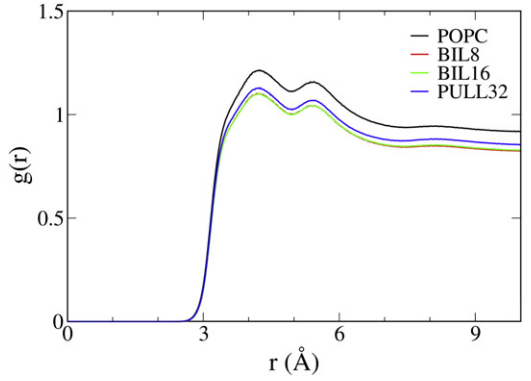

Fig. 5. The radial distribution function (RDF) of water around the choline group of POPC for all the different simulations.

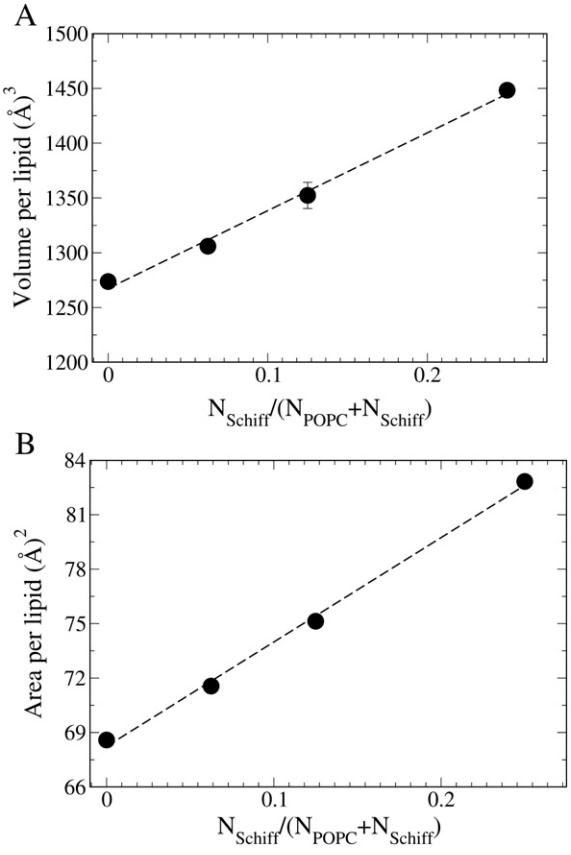

Fig. 7. The partial volume (A) and area (B) plotted as a function of the SCH mole fraction. The straight lines indicate that the SCH-POPC mixture is ideal, and the partial area of each entity can be calculated from the equation of the line (see text).

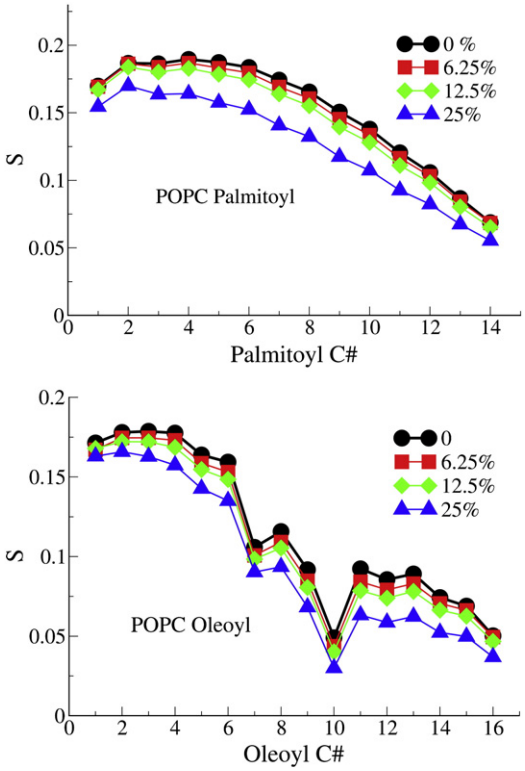

Fig. 8. Lipid tail order parameters for the POPC oleoyl and palmitoyl tails. The trends look similar for the POPE acyl tails, which are therefore not shown. There is a significant decrease in the order of parameters in the 25% SCH simulation only.

The presence of the putative Schiff bases in the phosphatidylcholine bilayer modifies the molecular and supramolecular lipid properties in a concentration-dependent manner. Pronounced changes are observed for membrane thickness which decreases, and average cross sectional areas of membrane phospholipids which increase upon increasing concentration of SCH. Effects on order parameters of fatty acyl chains are not very significant up to 12.5% SCH concentrations, beyond which the order parameters are significantly reduced. All these changes in membrane dynamics and packing are likely to change the native environment of functional membrane proteins. A decrease of membrane thickness will inevitably lead to a decrease in solubilization and as a consequence of conformational stability and/or the tilt of hydrophobic polypeptides. The reduction of membrane order parameters at high Schiff base concentrations may in addition give more conformational freedom for lipid-associated proteins thus modulating their activities.

Apart from alterations of membrane protein function, Schiff base formation between aldehydophospholipids and aminophospholipids is likely to local lipid arrangements in the bilayer and decrease bilayer stability. Depending on the aminophospholipid involved, these effects will be more or less pronounced. Phosphatidylethanolamine (PE) is a bulk phospholipid of all subcellular membranes of human cells. In the plasma membrane, it preferentially but not exclusively localizes to the cytoplasmic leaflet. Phosphatidylserine (PS) is much less abundant and preferentially localizes to the inner side of the surface membrane, too. So, if PE is modified to a large extent, this will affect the physical properties of large membrane areas. Chemical reaction of PS with phospholipid aldehydes would imply also dramatic changes in cell–cell communication. PS is exposed during OXPL-induced apoptosis on the cell surface where it is an "eat me signal" for the macrophages to phagocytose the dying cells [30]. Masking the PS head group could prevent the damaged cells from being cleared by this mechanism.

The physiological consequences of Schiff base formation between PS or PE and phospholipid aldehydes maybe of extraordinary consequence. The SCHs will result in impaired recognition by lipid metabolizing enzymes and changes in protein-mediated lipid transport or membrane–membrane interactions (see above). The consequences for membrane organization, which modulates lipid biochemistry and membrane stability, may be dramatic as well. It can be inferred from the putative chemical structure of a phospholipid Schiff base (Fig. 1) that the cross sectional area of its hydrophobic domains is roughly the same as the cross section of its polar head group. Thus, it might look like an oblate cylinder with three tails because of the long spacer in the head group. Cylindrical molecules (*e.g.* phosphatidylcholine) stabilize bilayer structures. In contrast, "inverted cones" such as phosphatidylethanolamine increase the tendency of a lipid assembly to form nonbilayer, *e.g.* hexagonal, phases [31]. Such an effect is potentiated by the space filling polyunsaturated fatty acids, which are frequently bound to the *sn*−2 positions of the latter phospholipid. As a consequence, these compounds may favor processes that are facilitated by high curvature such as membrane fusion and vesiculation. Membrane vesiculation or blebbing is a hallmark of apoptosis. Oxidized phospholipids including phospholipid aldehydes massively induce apoptosis in (vascular) cells, *e.g.* by formation of downstream ceramides [29]. Schiff base formation would counteract the release of membrane particles that possess high biological activity and propagate the toxic lipid effects *via* the circulation to sites far distant from where they originate [30]. This assumption is in line with the observation that POVPC causes much less membrane blebbing in cultured macrophages than the analogous PGPC [32]. The latter OXPL contains a carboxyacyl instead of an aldehydoacyl chain in position *sn*−2 of glycerol, which cannot chemically react with aminophospholipids.

Of course, the hypotheses outlined above are solely based on molecular simulations of phospholipid Schiff bases and must be subject to experimental verification. First of all, sufficient amounts of chemically defined reference compounds must be synthesized for analytical and functional studies. Secondly, the putative lipid–lipid adducts must be identified and quantified in biological samples of cells, tissues and organs. The methods of mass spectrometry are around to meet this challenge. Finally, the physical properties of these compounds in artificial membranes have to be characterized and their roles in pathophysiology have to be demonstrated.

Acknowledgements

MEMPHYS—Center for Biomembrane Physics is supported by the Danish National Research Foundation. The computations were done at the SDU node of the Danish Center for Scientific Computing (DCSC). HK is funded by a Lundbeck Junior Group Leader Investigator Fellowship. We thank Jacob Kongsted for the QM/MM calculations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2013.03.030.

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